I. Status of the Claims

Claims 1, 2, 4-7, 9-20 remain in this application. Claims 3 and 8 have been cancelled. Claims 1, 2, 5, 7, and 10 have been amended.

II. Rejections under 35 U.S.C. § 112, 1st Paragraph

Claims 1-11 stand rejected under 35 U.S.C. § 112, 1st paragraph, as not being enabling for cancers as enumerated in the Action. The independent claims 1 and 7 have been amended to restrict the scope of cancer cells to "myeloma cancer cells." Applicants note that the Action acknowledges that the application is enabling as to myeloma, thus rendering the rejection moot. Applicants respectfully request that the rejection as applied to those claims remaining in the case, i.e., 1, 2, 3-6, and 8-10, be reconsidered and withdrawn.

III. Rejections under 35 U.S.C. § 103(a)

Claims 1-20 are rejected as unpatentable as being obvious over Hirano et al., U.S. patent No. 6,020,363, in regard to isocoumarin compounds being useful in the treatment of malignant tumors, and particularly in view of in view of DiPiro et al., A Pathophysiologic Approach to Pharmacotherapy, 1989, pp. 1345, 1346 and 1496 in regard to teaching the co-administration of various chemotherapeutics. The Action states "it would have been obvious to the one having ordinary skill in the art to combine these prior art teachings in order to arrive at the instantly claimed composition of isocoumarin derivatives with other chemotherapeutic agents." Applicants respectfully traverse.

The pending claims are now limited to myeloma. The first section of text in Hiano et al. specifically cited in the Action merely recites "malignant tumors" in a list of disease primarily associated with an abnormality in immunological regulatory function:

> The above-described compounds of the present invention and pharmaceutically acceptable salts thereof have an inhibitory effect, for example, on collagen-induced arthritis, as will be more fully described later. Accordingly, they are considered to be

> useful for the prevention and treatment of diseases associated primarily with an abnormality in immunological regulatory function, including autoimmune diseases such as chronic rheumatism, systemic lupus erythematosus, systemic scleroma, periarteritis nodosa, ulcerative colitis and juvenile diabetes; **malignant tumors**; severe infectious diseases; and the like. (Hirano et al., col. 7, l. 13-25, emphasis added).

Thus, this disclosure teaches the use of the isocoumarin of the present invention for use in the genus of all malignant tumors, incorporated as a subgenus in the larger subgenus of all diseases primarily associated with an abnormality in immunological regulatory function based on activity in a murine arthritis model.

The second section of text in Hiano et al. specifically cited in the Action, when placed in context, recites "malignant solid tumors" in a list of diseases primarily associated with vascularization:

> Moreover, they also have an inhibitory effect, for example, on vascularization induced by tumor cells in the mouse back subcutaneous transplantation method. Accordingly, they are considered to be applicable to the prevention and treatment of diseases associated primarily with vascularization, such as the growth and metastasis of **malignant solid tumors**, diabetic retinopathy, various chronic inflammatory diseases, psoriasis, vascularization accompanying keratoplasty, and arteriosclerosis (Hirano et al., cl. 7, l. 26-34, emphasis added)

Myeloma is a cancer of the bone marrow, and since "solid tumors" are generally defined as cancer of body tissues other than blood, bone marrow, or the lymphatic system, the phrase "malignant solid tumors" would generally not be understood to teach a use for myeloma.

The first section of text cited in DiPiro et al (pp 1354 and 1355), are directed at combination chemotherapy regimens in metatstaic breast cancer, and thus are inaposite for citing against myeloma. The second section of text cited in diPro (p. 1496), teaches the use of glucocorticoids for the treatment of hypercalcemia resulting from myeloma and teaches no use of combinations of glucocorticoids for causing death of myeloma cancer cell.

The cited references do not teach not provide direction nor motivation for one or ordinary skill in the art to combine the isocoumarin derivative NM-3 and a glucocorticoid as specified in the pending claims for use of treating myleoma or killing or inhibiting the proliferation of myeloma cells. They do teach the use of the isocoumarin of the present claims for malignant tumors and, although the cited reference does not provide such information, Applicants recognize that glucocorticoids and the other specified agents other than NM-3 are used for the treatment of myeloma, in fact that is the specific reason they were included in the claims. Applicants understand that the bases of the alleged prima facie obviousness upon which the current rejections are based, is the generic teaching of Hirano et al of the use of NM-3 for malignant tumors being combined with other known agents used for myeloma. Applicants respectfully submit that such an alleged prima facie case of obviousness is defeated by the surprising results set forth in the present application.

Although Hirano et al. teaches two bases for the use of isocoumarins for treatment of tumor, i.e., immunomodulation and effects on vascularization, recent publications have focused on the antiangiogenic effects of NM-3 (see Reimer et al., Cancer Res., 62:789-795, 2002 (ref. C11 in IDS) and Salloum et al., Cancer Res. 60:6958-6963, 2000 (ref. C8 in IDS)). The target cell population for inhibiting neovascularization is primarily endothelial cells. NM-3 has been noted to have a selective toxicity towards endothelial cells as opposed to tumor cells (Salloum et al., reporting toxicity NM-3 to human endothelial cells but not murine Lewis lung carcinoma or Seg-1 human esophageal adenocarcinoma; Reimer et al., selective toxicity towards human endothelial cells as opposed to human MCF7 and MDA-MB435 breast tumor cells, human HT-29 colon tumor cells and human MKN28 cells gastric tumor cells). In fact, the in vivo effects of combinations of NM-3 and chemotherapeutic agents have been postulated to be via the effect on endothelial cells (Reimer et al., at page 794: "Results of in vitro assays of angiogenesis suggest that the effects of combining NM-3 with

chemotherapeutic agents in causing TGI [tumor growth inhibition] are mediated through decreased proliferation of endothelial cells").

Surprisingly, Applicants have found that NM-3 has effects directly on human myleoma cells lines. In MM1.S cells, NM-3 potentiates dexamethasone induced cell death in a dose dependent manner, but not doxorubicin mediated cell death (application page 11, line 7 - page 30, line 10 and FIG. 10). In RPM18226 and U266, NM-3 alone resulted in a dose dependent decrease in cell viability in addition to augmenting the effects of dexamethasone induced cell death (page 39, line 11 - page 40, line 4.

The initial studies regarding the role of angiogenesis and the potential for antiangiogenic therapeutic agents had been conducted using solid tumors. At the time of filing the instant application, February, 2002, the role of angiogenesis in hematological tumors, was becoming appreciated. This is shown in regard to multiple myeloma in the following quotes from articles appearing around the time of the filing date of the instant application. Rajkumar & Kyle, "*Angiogenesis in Multiple Myeloma*" Seminars in Oncology, 28:560-564, December 2001 (attached as Appendix 1), concluded a review article by stating: "Angiogenesis is increased in myleoma and **may** have importance in the pathogenesis of plasma cell disorders." (page 563, emphasis added). Yang & Han, "*Angiogensis in Hematologic Malignacies and its Clinical Implications*," Int. J. Hematol., 75:246-256, 2002 (accepted for publication November 2001, attached as Appendix 2), stated that: "Taken together, thus far there are 3 lines of evidence in studies of MM [multiple myeloma] that **suggest** a role for angiogenesis factors in the regulation of tumor cell growth and disease activity." (page 250, emphasis added). Some of the impetus to implicate angiogenesis in multiple myeloma was the clinical effectiveness of thalidomide, a compound known to inhibit angiogenesis. However, at the time of filing the mechanism of action of thalidomide in multiple myeloma was unclear, as indicated by Zweegman & Huijengs, "Treatment of Myeloma: Recent Developments," Anti-Cancer Drugs, 13:339-351, April 2002, (attached as Appendix 3) commenting of previous studies: "The efficacy of thalidomide [in

multiple myeloma] appeared not to be solely the result of inhibiting angiogenesis, as no correlation was found between microvessel density and response to therapy." And also: "In summary, thalidomide has proven to be effective in relapsed and refractory multiple myeloma, although the mechanism is still unclear." (page 343).

Thus, at the time of filing, NM-3 was known as an antiangiogenesis agent with a selective toxicity towards endothelial cells and the implications of angiogenesis in the pathology of multiple myeloma were neither as established nor understood as those for solid tumors. Thus Applicants contend that the selection of the cancer species multiple myeloma as a target cancer for NM-3 from the genus of all possible cancers was not obvious to one of ordinary skill in the art as the time of filing. Further, the pending claims require a combination with a glucocorticoid. Applicants contend that a finding of an additive effect of the combination of NM-3 and a glucocorticoid **directly** on the multiple myeloma cancer and implicates an activity other than antiangiogenesis is surprising. Applicants respectfully submit that the forgoing provides the basis for reconsideration and withdrawal of the pending claims as being obvious over Hirano et al., U.S. patent No. 6,020,363, alone or in view of DiPiro et al.

IV. Agata et al., 2001

Applicants note that Agata et al., "*NM-3, a Novel Angiogenesis Inhibitor, Potentiates Dexamaethasone-Induced Apopotsis in Multiple Myeloma Cells,*" Proceedings of the 2001 AACR-NCI-EORTC International Conference, p67 October 2001 (IDS reference C9), was not cited in the Action. Applicants respectfully submit that they stand ready to submit a declaration under 37 CFR § 1.132 as provided by *In re Katz*, 215 USPQ 14 (CCPA 1982), if so required to remove this publication as a reference under 35 U.S.C. § 102(a).

V. Conclusion

In light of the foregoing, Applicants respectfully submit that the claims are in condition for allowance, and an early notification to that effect is earnestly solicited. Should Examiner Jones have any questions regarding this response, a telephone call to the undersigned is invited. Please date stamp the enclosed postcard as evidence of receipt.

Respectfully submitted

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New Approaches to the Treatment of Multiple Myeloma

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Angiogenesis in Multiple Myeloma

S. Vincent Rajkumar and Robert A. Kyle

Angiogenesis is the process of new blood vessel formation, and normally occurs during embryonal growth, wound healing, and the menstrual cycle. It is essential for the proliferation and metastases of most malignant neoplasms. There is now growing evidence that angiogenesis is increased and is likely important in multiple myeloma . Recent evidence suggests that angiogenesis is greater in multiple myeloma compared to monoclonal gammopathy of undetermined significance (MGUS). Angiogenic cytokines such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are expressed by neoplastic plasma cells, and may play a role in the increased angiogenesis seen in myeloma. In a study of 400 patients with plasma cell disorders, microvascular density (MVD) was significantly higher in smoldering myeloma, newly diagnosed myeloma, and relapsed myeloma compared to controls, MGUS, and primary amyloidosis. In another study involving 74 newly diagnosed patients with myeloma treated at the Mayo Clinic, overall survival was significantly longer in patients with low-grade angiogenesis compared to those with high-grade or intermediate-grade angiogenesis. The finding of increased angiogenesis in myeloma provides the rationale for the study of antiangiogenic therapy in this disease.

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ANGIOGENESIS is the formation of new blood vessels, and occurs physiologically during embryonal growth, wound healing, and in the female genital system during the menstrual cycle. Angiogenesis is also important for the proliferation and metastases of most malignant neoplasms.[1] In the absence of angiogenesis, tumors cannot grow beyond 1 to 2 mm in size.[1] Increased angiogenesis has been found to be an adverse prognostic factor in several solid tumors.[2,3] Although many initial studies were performed on solid tumors, recent evidence indicates that angiogenesis is increased and may be important in hematologic malignancies as well.[4,5] This review will summarize the studies conducted by us to examine angiogenesis in myeloma and related plasma cell disorders.

Multiple myeloma is a plasma cell proliferative disorder that accounts for 1% of all cancers and slightly more than 10% of all malignant hematologic neoplasms.[6] It is characterized by the presence of a monoclonal (M) protein, lytic bony lesions, and increased plasma cells in the bone marrow, and it may be associated with anemia, renal failure, and hypercalcemia.[7] Not all patients with an M protein have multiple myeloma. Asymptomatic patients with an M protein level less than 3 g/dL, bone marrow plasma cells less than 10%, and no anemia, renal failure, lytic bone lesions, or hypercalcemia are considered to have monoclonal gammopathy of undetermined significance (MGUS).[8] Approximately 20% to 25% will eventually transform to overt myeloma, amyloidosis, or non-Hodgkin's lymphoma at a rate of 1% per year.[9]

Some patients have a serum M protein level that is ≥3 g/dL and/or ≥10% plasma cells in the bone marrow, without anemia, bone lesions, hypercalcemia, or renal insufficiency, and are considered to have smoldering multiple myeloma.[10] These patients have a higher risk of transformation to myeloma than those with MGUS. However, many patients with smoldering myeloma can be observed without therapy for months to years.

Patients with primary amyloidosis with ≥30% plasma cells in the bone marrow can be considered to have myeloma in addition to amyloidosis. Table 1 summarizes the criteria used in the definition of plasma cell disorders.

MEASUREMENT OF BONE MARROW ANGIOGENESIS

With the exception of lymphomas and solitary plasmacytomas, measurement of angiogenesis in hematologic malignancies such as myeloma depends on the evaluation of microvessels in bone marrow core biopsy samples. The extent of bone marrow angiogenesis in myeloma is assessed using standard immunohistochemical stains, such as von

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Table 1. Mayo Clinic Criteria for the Diagnosis of MGUS, Smoldering Myeloma, and Multiple Myeloma

MGUS	Serum M protein < 3 g/dL *and* bone marrow plasma cells < 10% *and* absence of anemia, renal failure, hypercalcemia, and lytic bone lesions
SMM	Serum M protein ≥ 3 g/dL *and/or* bone marrow plasma cells ≥ 10% *and* absence of anemia, renal failure, hypercalcemia, and lytic bone lesions
MM	Presence of a serum/urine M protein, bone marrow plasmacytosis *and* anemia, renal failure, hypercalcemia, or lytic bone lesions; patients with primary systemic amyloidosis *and* ≥30% bone marrow plasma cells are considered to have both multiple myeloma and primary amyloidosis

Abbreviations: MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering multiple myeloma; IMM, indolent multiple myeloma; MM, multiple myeloma.

Willebrand factor (vWF; factor VIII– related antigen) or CD34, to identify bone marrow microvessels.[11-13] In our laboratory, immunohistochemical staining of microvessels is performed using a labeled streptavidin-biotin peroxidase method, as previously described, on a Ventana ES automated immunohistochemistry stainer (Ventana Medical Systems, Tucson, AZ) using buffers and detection reagents supplied by the manufacturers.[4] For vWF staining, deparaffinized tissue sections are subjected to protease digestion on the instrument with protease 2 for 12 minutes prior to immunostaining. The primary antibody (DAKO A0082, Dako, Carpinteria, CA; diluted 1:2,000) is incubated with tissue sections for 24 minutes. For CD34 staining, deparaffinized tissues are pretreated using EDTA (pH 8.0) in a steamer for 30 minutes followed by a cool down for 5 minutes. The primary antibody (monoclonal CD34, Becton-Dickinson, San Diego, CA; diluted 1:10) is incubated with the tissue sections for 32 minutes. For both stains, the aminoethyl carbazole (AEC) detection kit (Ventana Medical Systems) is used for antigen visualization; sections are counterstained with a light hematoxylin and then coverslipped with Kaiseri's glycerol jelly (Mayo Medical Laboratories, Rochester, MN). Paraffin sections of well-vascularized tonsil are run with each batch to serve as a positive control, and a section stained with nonimmune rabbit immunoglobulin is used as a negative control for each sample tested.

The commonly used quantitative methods to evaluate microvessels identified by immunohistochemical stains are: simple grading (low, intermediate, high), determination of microvessel density (MVD), and estimation of the percentage of tissue surface area that is occupied by microvessels (MSA).[11,12] All estimations should be done in a blinded manner. Methods used in our laboratory are detailed below, and are similar to standard methods used by other investigators.[4,14,15]

For simple grading, slides are scanned at 100x, 200x, and 400x magnification, and based on the extent of microvessel staining, each slide is assigned an angiogenesis grade: low, intermediate, or high. This grading was based solely on visual assessment of the number of microvessels. The entire stained sample was considered when assigning the angiogenesis grade.

For MVD estimation each slide is first scanned at 100x magnification to determine three "hot spots," defined as areas with the maximum number of microvessels. The hot spots are then examined at 400x magnification, using a 10x ocular and 40x objective lens. Microvessels are counted in each of the three hot spots at 400x magnification. Large vessels and vessels in the periosteum or bone are excluded. Areas of staining with no discrete breaks are counted as a single vessel. Presence of a lumen is not required. MVD is estimated by determining the average number of vessels in each of the three hot spots and expressing the result as number of vessels per 400x high-power field.

Computerized image analysis is used for estimation of MSA. Hot spots are evaluated under 200x magnification and captured using a digital camera. Optimas 6.0 software for Windows 95 (Optimas Corp., Seattle, WA) is used for analysis of digitally captured images. Using computerized pixel counting, MSA is determined and expressed as the percentage of the captured region of interest that is occupied by immunohistochemical staining.[16] An optimized MSA value can be determined by eliminating the area occupied by fat and expressing the result as a percentage of cellular area occupied by staining.[13,14]

ANGIOGENESIS IN MULTIPLE MYELOMA

Increased Angiogenesis in Myeloma

Vacca et al were the first to demonstrate that increased bone marrow angiogenesis occurs in

Table 2. Bone Marrow Angiogenesis in Plasma Cell Disorders

Group	Median MVD per 400× Field (range)	High Angiogenesis Grade (%)
Normal (n = 42)	1.3 (0-11)	0
Primary amyloidosis (n = 87)	1.7 (0-10)	0
MGUS (n = 76)	3 (0-23)	1
Smoldering multiple myeloma (n = 112)	4 (1-30)	3
Newly diagnosed myeloma (n = 99)	11 (1-48)	29
Relapsed myeloma (n = 26)	20 (6-47)	42

marrow samples from patients with multiple myeloma compared to those with MGUS.[11] The extent of angiogenesis was correlated to the plasma cell labeling index (PCLI; a measure of plasma cell proliferative activity) and with disease activity. Vacca also demonstrated the angiogenic ability of myeloma cells in an in vitro chick embryo chorioallantoic membrane (CAM) angiogenesis assay,[17] with a linear correlation between the angiogenic activity and marrow vascularization estimated by analysis of microvessels.

We have recently completed the first large study of bone marrow angiogenesis in the various stages of plasma cell disorders. Bone marrow samples from 400 patients with plasma cell disorders seen at the Mayo Clinic were studied: MGUS (76 patients), smoldering myeloma (112 patients), newly diagnosed untreated myeloma (99 patients), relapsed myeloma (26 patients), and amyloidosis (87 patients).[18] Bone marrow angiogenesis was studied in a blinded manner using immunohistochemical staining for CD34 to identify microvessels. The median (range) MVD per 400x field was 1.3 (0 to 11) in the controls, 1.7 (0 to 10) in amyloidosis, 3 (0 to 23) in MGUS, 4 (1to 30) smoldering myeloma, 11 (1to 48) in untreated myeloma, and 20 (6 to 47) in relapsed myeloma, $P < .001$ (Table 2). MVD was significantly higher in smoldering myeloma, untreated myeloma, and relapsed myeloma compared to controls, MGUS, and amyloidosis, $P < .001$; MVD was not significantly different between controls and amyloidosis. MVD was significantly higher in relapsed myeloma compared to new, untreated myeloma ($P = .02$). High-grade angiogenesis was present in a significantly greater proportion of patients with newly diagnosed myeloma and relapsed myeloma, compared to the other groups, $P < .001$ (Table 2). This study clearly shows the progressive increase in angiogenesis from MGUS to smoldering multiple myeloma to active multiple myeloma to relapsed myeloma, suggesting a role for angiogenesis in the pathogenesis of myeloma.

Prognostic Value of Angiogenesis in Myeloma

We performed a study in conjunction with the Eastern Cooperative Oncology Group (ECOG) that demonstrated that high angiogenesis grade and increased MVD conferred a poor outcome in newly diagnosed myeloma. Seventy-four newly diagnosed patients with myeloma treated at Mayo Clinic, on two ECOG trials (E9486 and S9321) were studied. Overall survival was significantly longer in patients with low-grade angiogenesis (53 months) compared to patients with high-grade (24 months) or intermediate-grade angiogenesis (48 months), $P = .018$.[19] In a separate study of 211 patients with untreated myeloma and smoldering myeloma, bone marrow MVD was a prognostic factor for survival, confirming our earlier observation. Survival was 28 months in smoldering myeloma/untreated myeloma with high-grade angiogenesis, compared to 53 months for those with low-grade angiogenesis, $P = .02$.[18] On multivariate analysis, despite being highly correlated to the PCLI, MVD appeared to have a trend toward independent prognostic value ($P = .04$).

Effect of Treatment on Bone Marrow Angiogenesis in Myeloma

Although it was clear that increased bone marrow angiogenesis occurred in active myeloma, it was not known whether this resolved with effective therapy, such as high-dose therapy with stem cell support. We studied this question by estimating bone marrow angiogenesis before autologous stem cell transplantation and at the time of response in 13 patients with myeloma (seven complete and six partial responders).[4] Baseline MVD was significantly different between patients with myeloma and normal controls, $P = .001$. After transplantation, MVD continued to be high compared to controls, $P = .003$. There was no difference in MVD at the time of complete or partial response compared to values before transplantation. This study demonstrated that marrow angio-

genesis is increased in myeloma compared to normal bone marrows and that it persists even after stem cell transplantation.

A subsequent study has now shown that angiogenesis as measured by conventional methods does not change significantly after conventional-dose therapy in myeloma.[20] These studies provide the rationale for exploring antiangiogenic strategies as maintenance therapy in myeloma, since the residual microvessels may provide a good milieu for the residual myeloma cells to proliferate and contribute to relapse.

Correlation of Increased Angiogenesis in Myeloma to Plasma Cell Proliferation

Increased angiogenesis in myeloma is associated with increased plasma cell proliferation measured by the PCLI. In the ECOG newly diagnosed myeloma study (74 patients), bone marrow MVD was significantly correlated with the PCLI.[19] We have recently confirmed this in a separate larger study as well.[18]

ANTIANGIOGENIC THERAPY IN MYELOMA

The interest in tumor angiogenesis peaked in the last 3 years with the discovery of angiostatin and endostatin antiangiogenic therapy.[21,22] The concept represents a paradigm shift in that cancer therapy in this situation targets the tumor blood supply rather than the tumor itself. Further since the targets (endothelial cells) are normal nonmalignant cells, they will probably be less likely to acquire drug resistance unlike cancer cells. With the discovery of increased angiogenesis in myeloma there has been a significant interest in testing antiangiogenic agents in this disease.

Thalidomide in Myeloma

Based on its antiangiogenic properties in animal models and in vitro systems, and its availability for clinical trials, thalidomide has become a natural choice for initial antiangiogenesis trials. Studies at the University of Arkansas and other institutions have now confirmed its activity in relapsed myeloma.[23-26] Based on the evidence so far, thalidomide can be recommended for relapsed myeloma, although the Food and Drug Administration has not yet approved the agent for this indication. Studies are ongoing to determine the efficacy of thalidomide when combined with other effective agents for myeloma such as dexamethasone,[27] and combination chemotherapy.[28]

Clinical Trials With Other Antiangiogenic Drugs in Myeloma

With the responses seen with thalidomide there is now interest in clinical trials with other new antiangiogenic agents in myeloma. We are currently evaluating 2-methoxyestradiol, a metabolite of estrogen that has antiangiogenic properties,[29] as a potential therapeutic agent in myeloma. Novel analogs of thalidomide are also being developed, in an attempt to minimize the adverse effects while increasing clinical efficacy. One such analog is now undergoing phase I testing at the Dana-Farber Cancer Institute and the University of Arkansas. Phase II trials are planned to start in 2002.

CONCLUSIONS

Angiogenesis is increased in myeloma and may have importance in the pathogenesis of plasma cell disorders. It has prognostic value in myeloma. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are likely involved in the increased angiogenesis seen in myeloma. The role of VEGF as a paracrine growth factor for neoplastic plasma cells is being studied. Increased angiogenesis has provided a new therapeutic target in myeloma leading to studies with thalidomide and other antiangiogenic agents in this disease.

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Review Article

Angiogenesis in Hematologic Malignancies and Its Clinical Implications

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Abstract

Angiogenesis is defined as a neoformation of blood vessels of capillary origin. Hematopoiesis is closely linked with angiogenesis, for they share a common ancestor, the hemangioblast. Although it is well established that growth in solid tumors is dependent on angiogenesis, its role in hematologic malignancies has not yet been clarified. In this review, the direct evidence, ie, increased microvessel density, and the indirect evidence, ie, elevated level of angiogenic factors or overexpression of messenger RNA or protein of angiogenic factors, for and against the role of angiogenesis in the development and progression of hematologic malignancies are presented. *Int J Hematol.* 2002;75:246-256.

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Key words: Angiogenesis; Hematopoiesis; Prognosis; Angiogenic factor; Treatment

1. Introduction

Angiogenesis is defined as a neoformation of blood vessels of capillary origin. There are at least 2 different types of angiogenesis: sprouting and nonsprouting [1]. Angiogenesis is essential for reproduction, development, and repair, and it is a key step in tumor growth, invasion, and metastasis [1-6]. It is tightly regulated by a set of positive and negative angiogenic factors [5,6]. The major positive regulatory molecules are basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF); in addition, more than 20 different proteins have been shown to have angiogenic properties, such as angiogenin, acidic FGF, and hepatocyte growth factor (HGF) [5-14]. The major negative regulatory molecules that have been described include angiostatin, endostatin, thrombospondin, and platelet factor-4 [5]. These factors are thought to act in a paracrine or autocrine manner, however, the exact mechanism is unknown [15]. Angiogenesis has been found to be an unfavorable prognosis factor for solid tumors. So far, about 20 angiogenesis inhibitors have entered phase II or III clinical trials [16]. Although it is well established that growth in solid tumors is dependent on angiogenesis, the role of angiogenesis in hematologic malignancies has not yet been clarified. In this review, we will explore the role of angiogenesis in the development and progression of hematologic malignancies.

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2. Angiogenesis, Hematopoiesis, and Bone Marrow Microenvironment

There is a long-recognized close relationship between the development of blood and endothelium, suggesting that hematopoietic stem cells (HSCs) and endothelial progenitor cells may well share a common precursor [17-25]. A number of molecules, such as CD34 and KDR, are expressed in both endothelium and hematopoietic progenitors [26-29]. Furthermore, mice lacking the early endothelial marker, fetal liver kinases (Flk-1), display a defect in both hematopoietic cells and vasculature [30], and Flk-1 appears to play an important role in the formation of both lineages [31]. A major role of endothelial cells in the regulation of hematopoiesis was recently established in studies where Flk-1 and Flt-1 genes were disrupted in mice embryonic stem cells by a homologous recombination technique [30,32,33]. Both Flk-1 and Flt-1 (fms-like tyrosine kinase-1) play pivotal roles in endothelial development and

are expressed at high levels during early stages of embryonic angiogenesis and vasculogenesis [30]. These data suggest that early interaction between endothelial cells and hematopoietic stem cells may be critical for stem cell self-renewal and expansion.

There are 3 main cellular systems in the bone marrow (BM): hematopoietic, endothelial, and stromal. BM microvascular endothelial cells (BMECs) regulate proliferation of hematopoietic progenitor cells and long-term culture-initiating cells, in part by elaboration of lineage-specific cytokines, such as interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, and M-CSF [34]. Although constitutive production of soluble cytokines by BMEC monolayers may be essential for the lineage-specific proliferation of the progenitors, direct cellular contact is critical for the long-term preservation and expansion of progenitors [34]. In addition, vascular cell adhesion molecule-1, which was expressed by BMECs, and stromal cell–derived factor-1, which was secreted by BM fibroblast cells, played important roles in the homing of hematopoietic progenitor cells to BM [34,35]. Endothelial cells have been shown to produce many of the cytokines, which are known to play a role in the proliferation and differentiation of hematopoietic progenitors [36].

Thus far, much evidence suggests that angiogenic factors are hematopoietic growth factors and vice versa [37-47]. Also, current data have demonstrated that VEGF plays a role in regulating normal hematopoiesis [30,31,48-50]. VEGF is constitutively produced by human megakaryocytes and platelets, and its release can be induced by thrombin as well as by platelet activation [51,52]. Normal BM erythroblasts are a major source of VEGF-A in vivo, and BM stromal cells also can produce VEGF, which may act as a paracrine factor on monocyte/macrophages and/or endothelial cells [39,53]. Paracrine release of VEGF by progenitor cells and elaboration of hematopoietic growth factors by BM endothelium in vivo could result in a paracrine loop supporting proliferation of both hematopoietic progenitor cells and BM endothelial cells. HGF is constitutively produced by human and murine BM stromal cells and indirectly promotes hematopoiesis [54-56]. In addition, BM stromal cells are reported to express c-Met, and stromal cell lines respond with slight growth inhibition upon exposure to HGF, indicating autocrine inhibition by HGF on the stromal cells [54,56].

Regarding hematopoietic progenitor cells, interaction with endothelium by paracrine secretion of cytokines that specifically act on endothelial cells may be important in the regulation of proliferation and trafficking. Recent evidence suggests that HSCs are required for angiogenesis [43]. There is also evidence suggesting that temporal and regional activation of VEGF/VEGFR2 and Ang-1/Tie-2 signalling pathways is critical for mobilization and recruitment of HSCs and circulating endothelial progenitors and may play a role in the physiology of postnatal angiogenesis and hematopoiesis [50].

In summary, current data have suggested that endothelial cell growth and survival may contribute to the maintenance of the BM microenvironment and hematopoiesis. Hematopoietic stem/progenitor cells, in turn, can promote endothelial cell proliferation, migration, and tube formation.

3. Angiogenesis in Hematologic Malignancies

As mentioned above, it is well established that both the growth and metastasis of solid tumors are dependent on angiogenesis. However, the role of angiogenesis in hematologic malignancies has not yet been clarified. There is both direct and indirect evidence for and against the role of angiogenesis. The direct evidence comes from the observation of microvessel density (MVD), whereas the indirect evidence comes from detection of the level or expression of the messenger RNA (mRNA) or the protein of angiogenic factors, such as VEGF, bFGF, and HGF.

3.1. Angiogenesis in Leukemia

Prior to the identification of angiogenic factors, DeCarvalho [57] reported in 1978 that RNA from leukemic lymphocytes had in vitro angiogenic activity. Thus far, a mounting body of evidence has been accumulating that suggests a role for angiogenesis in the pathophysiology of leukemia. Elevated levels of angiogenic factors, such as VEGF, bFGF, and HGF, were revealed in leukemic cell lines, as well as in fresh blood and BM samples from acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), adult T-cell leukemia (ATL), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL) patients [58-85]. However, it should be kept in mind that the aforementioned angiogenic factors are multifunctional cytokines; therefore, the data should be interpreted with caution.

Determination of MVD is still the gold-standard method for assessment of angiogenesis. Perez-Atayde et al [59] evaluated BM biopsies from 40 children with newly diagnosed and untreated ALL. In 22 of the patients, angiogenesis after the completion of remission-induction chemotherapy was also evaluated. Biopsies from children with leukemia and from control subjects showed median MVD of 42 and 6 counts per field, respectively ($P \leq .0001$). MVD of the hot spots of leukemia specimens and control samples were also significantly different, 51 and 8 counts per field, respectively ($P \leq .0001$). In addition, urinary bFGF was measured in 22 of the children with newly diagnosed leukemia and in 39 healthy, age-matched control subjects. Urinary bFGF levels were increased in all 22 patients before treatment, were variable during induction chemotherapy, and demonstrated statistically insignificant decreases at the time of complete remission [59]. However, Schneider et al [60] reported that in 10 ALL patients, the level of urinary bFGF was not correlated with either the leukemic burden at diagnosis or the growth rate of the leukemia. On the contrary, they found that a normal bFGF urinary excretion might be associated with poor prognosis.

Fiedler et al [63] investigated the expression of VEGF and its receptors on fresh leukemic blasts. VEGF-specific transcripts were detected by polymerase chain reaction (PCR) in samples from 20 of 28 patients with de novo AML and in samples from 3 of 5 patients with secondary AML. Using immunocytochemistry, Fiedler et al found VEGF protein in 2 leukemic cell lines and in samples from 8 AML patients. Supernatants of fresh leukemic cells from 24 AML patients

contained significantly more VEGF than supernatants from BM cells of 9 healthy donors or of CD34-enriched cells from 3 healthy volunteer donors. Two receptors of VEGF, KDR and FLT1, were also detected in AML. Fiedler et al also investigated expression of FLT4 and its ligand VEGF-C in fresh samples from patients with AML. Using a sensitive PCR method, they detected FLT4 mRNA in 15 of 41 patients with de novo AML at diagnosis or relapse and in 3 of 12 patients with secondary AML, whereas FLT4 expression was not detected in BM samples from 15 healthy volunteer donors or in $CD34^+$ cells from 3 additional donors [64].

In a group of 99 patients with newly diagnosed AML, Aguayo et al [66] demonstrated that there was a relationship between increasing VEGF and shorter survival (P = .01), as well as shorter disease-free survival, both from the start of treatment and from the complete response date. In contrast, there was no relationship between VEGF level and white blood cell or blast counts or presence of an antecedent hematologic disorder; multivariate analysis indicated that VEGF was still prognostic for the above outcomes after accounting for these factors as well as treatment. In addition, Hayashibara et al [65] examined the prognostic significance of the VEGF level in ATL patients. No correlation was observed between VEGF level and survival time or ATL cell count. However, it is noteworthy that the incidence of extranodal involvement was closely correlated with a high VEGF level.

Using antibodies to CD34 as the endothelial marker, Kini et al [75] found that the number of BM microvessels increased in CLL patients and that the mean hot-spot MVD and the area with the highest MVD were significantly higher in CLL than in the control biopsy section. They also noted that urine bFGF level was elevated in CLL patients compared to that in control subjects. Using anti–factor VIII–related antigen (FVIIIrAg) antibody as endothelial marker, Aguayo et al [69] found that the vascularity was not increased in CLL patients compared to that in control subjects. Recent studies demonstrated that higher serum levels of VEGF were poor risk factors for the progression of disease in early B-cell CLL (B-CLL) [73]. Aguayo et al found that patients with lower intracellular levels of VEGF showed a trend toward shorter survival [76] and that CLL cells from patients with aggressive disease produce high levels of VEGFR-2 protein [77]. They also demonstrated the presence of 2 angiogenesis receptors, Flt1 and Tie1, in B-CLL, and that patients with early Rai stage disease that had higher levels of Tie1 had poorer survival rates [78]. In a recent report, Chen et al [74] demonstrated that CLL cells produce and secrete VEGF and that the predominantly expressed mRNA isoforms are VEGF121 and VEGF165. They found that VEGF is produced by both circulating and tissue-phase CLL cells. Using the chick embryo chorioallantoic membrane (CAM) assay, they demonstrated that CLL cell–secreted VEGF has angiogenic activity [74].

Recent studies have demonstrated that AML cells express VEGF as well as the VEGF receptors (VEGFR-1 and VEGFR-2) [63,64,70,72]. In addition, several leukemia cell lines were found to express the Flt-1 receptor for VEGF at a moderate-to-strong level [61]. Taken together, these findings suggest that VEGF may play the role of an autocrine growth factor for AML cells. Along these lines, VEGF was shown to protect AML cells from chemotherapy-induced apoptosis by up-regulating MCL1 (a member of the BCL2 family) [86]. Factors secreted by leukemic cells may also act in a paracrine manner on surrounding cells, for example, stromal or endothelial cells can be stimulated by 1 factor to produce other multiple hematopoietic growth factors, which in turn may affect the growth of leukemic blasts. Expanded endothelium resulting from paracrine stimulation by VEGF or bFGF has been postulated to play an important role in the pathogenesis and progression of human leukemias.

A significant amount of HGF was detected in blood and BM plasma of AML and CML patients, whereas in ALL patients, BM plasma HGF concentration remained low, within the range for healthy subjects [80,81]. The serum concentration of HGF was also elevated in AML patients [82]. These results suggest that some populations of myeloid-lineage cells have the ability to produce HGF. Recent studies showed that HGF and its receptor Met were overexpressed in some leukemia cases and in some leukemia cell lines [83-85].

Matrix metalloproteinases (MMPs) are a family of structurally and functionally related zinc-dependent endopeptidases consisting of at least 20 enzymes that are able to degrade all of the protein components of the extracellular matrix. Their activity is regulated at the levels of gene expression and of proenzyme activation and interaction with the tissue inhibitors of metalloproteinases (TIMPs) [87,88]. Correlation between high MMP expression and the increased metastatic potential of various human tumors have been documented [89]. Janowska-Wieczorek et al [90] compared the expression of gelatinases (MMP-2 and MMP-9) and TIMPs (TIMP-1 and TIMP-2) by leukemic cells to that by normal BM hematopoietic and stromal cells. All AML samples and leukemic cell lines that were tested expressed MMP-9 and/or MMP-2 mRNA and, accordingly, these gelatinases were secreted into media. Moreover, TIMP-1 and TIMP-2 mRNA and secreted proteins were demonstrated in all of the AML samples. Although all of the leukemic cell lines expressed TIMP-1, the HL-60 also expressed TIMP-2. In contrast, immature progenitor cells ($CD34^+$ cells) in normal steady-state BM did not express or secrete MMP-2 or MMP-9, whereas more mature mononuclear cells from normal BM expressed and secreted MMP-9. These findings suggest that these MMPs might be implicated in the invasive phenotype of AML.

Increased MVD was also found in the BM sections of CML patients [69,91] and patients with myelofibrosis (MF) [91,92]. Mesa et al [92] showed that in a group of 114 patients with MF, a group of 15 patients with polycythemia (PV), and a group of 17 patients with essential thrombocythemia (ET), 70%, 33%, and 12%, respectively, demonstrated increased MVD. The degree of increased angiogenesis in patients with MF might have an independent prognostic value. In addition to having increased MVD, leukemia patients have vessel architecture that is different from that of healthy subjects. Leukemic vessel architecture is tortuous and has increased

branching [91]. A recent study demonstrated that BM stromal cell bFGF expression in patients with chronic myeloproliferative diseases is abnormally decreased [93]. The mechanism of this abnormality is unknown. MVD is also increased in patients with myelodysplastic syndromes (MDS) [69,94]. Bellamy et al [95] demonstrated that monocytoid precursors in chronic myelomonocytic leukemia expressed VEGF in an intense cytoplasmic pattern with membranous coexpression of the Flt-1 or KDR receptors, or both. In AML and other MDS subtypes, intense coexpression of VEGF and 1 or both receptors was detected in myeloblasts and immature myeloid elements, whereas erythroid precursors and lymphoid cells lacked VEGF and receptor expression. Interestingly, Gunsilius et al [96] recently demonstrated that in CML patients both endothelial cells and pluripotent and committed hematopoietic progenitor cells contained the BCR/ABL fusion gene and therefore must have arisen from a common ancestor.

Activated endothelial cells can release a variety of cytokines that may stimulate leukemic cell growth [34,63]. Leukemic cells, in turn, have the capacity to release angiogenic factors, such as VEGF, and may also express related receptors, such as VEGFR-2, on their surfaces [62-64]. These findings suggest that leukemia cells induce angiogenesis in the BM and that leukemia might be angiogenesis dependent, raising the possibility for a role of antiangiogenic drugs in the treatment of leukemia.

3.2. Angiogenesis in Multiple Myeloma

As in leukemia patients, increased MVD was revealed in multiple myeloma (MM) patients (Table 1) [97-106]. Ribatti and colleagues demonstrated vivid angiogenesis and numerous mast cells in the BM of patients with active MM compared to that of patients with nonactive MM and to that of patients with monoclonal gammopathy of undetermined significance (MGUS) [97]. They also found that the risk of active disease in MM patients increased in parallel with the microvessel area and that BM angiogenesis was correlated with the plasma cell labeling index [98,99]. They found that U266 (MM cell line) cells secreted MMP-2, MMP-9, and bFGF. The conditioned medium of the U266 cell line stimulated cell proliferation and/or chemotaxis in cultured endothelial cells. U266 cells induced angiogenesis and mononuclear cell recruitment in the murine Matrigel sponge model and in a chick embryo CAM assay [61]. In another

Table 1.

Angiogenesis in Hematologic Malignancies*

Disease	No. of Patients	Endothelial Markers	MVD	Prognosis	Reference
AML	30	FVIIIrAg	Increased	NA	[69]
AML	64	vWF, TM	Increased	NA	[68]
AML	20	vWF, ULEX-E	Increased	NA	[67]
ALL	40	FVIIIrAg, CD31, CD34	Increased	NA	[59]
ALL	20	FVIIIrAg	Increased	NA	[69]
CML	24	FVIIIrAg	Increased	NA	[69]
CML	9	FVIIIrAg, CD31, ULEX-E	Increased	NA	[91]
CLL	12	FVIIIrAg, CD31, CD34	Increased	NA	[75]
CLL	23	FVIIIrAg	Normal	NA	[69]
MDS	32	FVIIIrAg	Increased	NA	[69]
MDS	82	FVIIIrAg	Increased	NA	[94]
MF	114	CD34	Increased	Poor	[92]
MF	6	FVIIIrAg, CD31, ULEX-E	Increased	NA	[91]
MM	46	FVIIIrAg	Increased	NA	[98]
MM	24	FVIIIrAg	Increased	Poor	[97]
MM	34	CD34	Increased	NA	[105]
MM	30	CD34	Increased	NA	[101]
MM	74	vWF	Increased	Poor	[102]
MM	44	CD34	Increased	Poor	[103]
MM	75	CD34	Increased	NA	[104]
MM	41	FVIII	Increased	NA	[114]
MGUS	15	CD34	Increased	NA	[101]
MGUS	21	FVIIIrAg	Increased	NA	[98]
WM	7	CD34	Increased	NA	[101]
B-NHL	88	FVIIIrAg	Increased	Poor	[122]
B-NHL	71	FVIIIrAg	Increased	Poor	[114]
B-NHL	72	FVIIIrAg	Increased	Poor	[126]
CBCL	18	FVIIIrAg, CD31	Increased	Poor	[125]
DLBC	36	FVIIIrAg	Increased	Poor	[128]
Mycosis fungoides	57	FVIIIrAg	Increased	Poor	[129]

*MVD indicates microvessel density; AML, acute myeloid leukemia; FVIIIrAg, factor VIII–related antigen; NA, not available; vWF, von Willebrand factor; TM, thrombomodulin; ALL, acute lymphocytic leukemia; ULEX-E, Ulex europeus I; CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndrome; MF, myelofibrosis; MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance; WM, Waldenström's macroglobulinemia; B-NHL, B-cell non-Hodgkin's lymphoma; CBCL, cutaneous B-cell lymphoma; DLBC, diffuse large B-cell lymphoma.

investigation; increased angiogenesis was found in myeloma BM prior to transplantation; however, no significant decrease in MVD posttransplantation was observed, even in the setting of complete response [100]. As to the prognostic value of BM angiogenesis in MM, there is no consensus of results. Some investigations suggest that increased MVD is a predictor of poor survival in newly diagnosed MM [102,103], whereas 1 study shows that although MVD is significantly increased in MM patients compared to that in control subjects, it is not correlated with overall survival [104].

Current data demonstrate that the levels of angiogenic factors, such as VEGF, bFGF, and HGF, are also increased in MM patients [107-114]. Sezer et al [107] reported that MM in stages II and III is associated with an increase in serum bFGF concentrations and that effective chemotherapy is accompanied by a significant decrease in the angiogenic factors bFGF, VEGF, and HGF, whereas no decrease of these factors could be found in nonresponders. Another group found that the concentrations of bFGF, VEGF, and HGF in MM patients were higher in BM than in peripheral blood [108], indicating that the BM environment is the major source of angiogenic factors. In a group of 398 patients with MM, serum HGF levels were analyzed at diagnosis and in serial samples from 29 patients. HGF was elevated at diagnosis in 43% of MM patients compared to that in healthy control subjects [111]. HGF and c-met mRNA were expressed in serum samples of 13 patients with MM. HGF was detected in the supernatants of 17 of 20 primary cultures of myeloma cells, whereas BM mononuclear cells from healthy control subjects did not produce detectable amounts of HGF [112]. Given the effect of HGF on angiogenesis, it is likely that HGF is 1 of the cancer-derived agents that induce vessel development in MM [113].

One study demonstrated that the progression of MM is accompanied by an increase in BM neovascularization [114]. This increase is paralleled by increased angiogenic and invasive potential of BM plasma cells, dependent, at least in part, on FGF2 and MMP-2 production. Induction of angiogenesis and secretion of MMPs by plasma cells in active MM may play a role in their medullary and extramedullary dissemination, raising the hypothesis that angiostatic/anti-MMP agents may be used for therapy in treating MM [114]. In addition, angiogenesis was demonstrated in both in vitro and in vivo models. In a SCID-hu mouse model, active angiogenesis was revealed in areas of myeloma cell infiltration, and the newly formed endothelial cells were of human origin [115]. Dominici et al [116] reported that in an in vitro endothelial colony assay (colony-forming units-endothelial [CFU-En]) a 5-fold higher number of CFU-En were found in MM cell cultures than in MGUS or control cultures. They also found that the numbers of CFU-En in cultures from MM specimens obtained at diagnosis or during disease progression were, respectively, 7-fold and 6-fold higher than those in MGUS cultures.

Taken together, thus far there are 3 lines of evidence in studies of MM that suggest a role for angiogenic factors in the regulation of tumor cell growth and disease activity. First, BM angiogenesis parallels disease progression and predicts poor outcome. Second, the marrow microenvironment supports both the growth of myeloma cells and the neovascularization of areas with myeloma cell infiltration. Third, myeloma cells express both angiogenic activity and angiogenic cytokines.

3.3. Angiogenesis in Lymphoma

Significant expression of VEGF transcripts was observed in Hodgkin's disease and peripheral T-cell lymphomas, particularly of the angioimmunoblastic type. In contrast, expression of VEGF was minimal or absent in follicle center lymphoma and B-CLL [117]. It is suggested that VEGF may be involved in the induction of the angiogenesis of both peripheral T-cell lymphomas and Hodgkin's disease, but not in low-grade B-cell lymphoma. Salven et al [118] measured serum VEGF (sVEGF) by enzyme-linked immunosorbent assay (ELISA) from sera taken from 82 patients with non-Hodgkin's lymphoma (NHL), before treatment, and stored for 9 to 15 years at −20°C. A higher than the median sVEGF level was associated with a poor World Health Organization performance status and a high International Prognostic Index. Patients with lower levels of sVEGF at diagnosis had a 71% 5-year survival rate compared to only 49% among those with a higher level of sVEGF. Salven et al also measured bFGF by ELISA from sera taken from 160 NHL patients before treatment. Serum bFGF concentrations (S-bFGF) ranged from undetectable to 34.7 pg/mL (median, 3.3 pg/mL). S-bFGF was detectable with a similar frequency in all subtypes of NHL. A high pretreatment S-bFGF was associated with poor overall survival. The 5-year survival rate for the patients within the highest quartile of S-bFGF concentrations (S-bFGF = 5.5 pg/mL) was only 39%, in contrast to a 60% survival rate for the patients with lower S-bFGF (P = .019). A high S-bFGF (within the highest quartile) was associated with poor outcome also in large-cell diffuse and immunoblastic lymphomas (5-year survival rates of 28% and 56%, respectively; P = .027), both of which composed the largest histologic group (n = 66) within the series. In multivariate analyses, S-bFGF was an independent prognostic factor, both when the highest quartile was used as a cut-off value (P = .0079) and when S-bFGF and other parameters were entered into the model as continuous variables (P = .024) [119]. Recently, the same research group reported that simultaneous elevation in serum levels of VEGF and bFGF is an independent predictor of poor prognosis in NHL [120]. These findings are supported by other researchers [121].

Current data demonstrate that MVD is increased in patients with lymphoma (Table 1). Vacca et al [99] reported that the microvessels were significantly more numerous in intermediate- and high-grade B-cell NHL (B-NHL) than in low-grade B-NHL. They subdivided the intermediate-grade cases into follicular and diffuse, and increased angiogenesis was observed in the latter (microvessel number, 12 ± 3 and 9 ± 2, respectively). The number of vessels tended to be higher in the high-grade than in the intermediate-grade B-NHL. In addition, Vacca et al reported that cells from lymphoma cell lines secreted the active form of MMP-2 and MMP-9 and also secreted both bFGF and VEGF. The cells

induced angiogenesis and mononuclear cell recruitment in the murine Matrigel sponge model and in a chick embryo CAM assay [61].

In a study of 88 patients with B-NHL, Ribatti et al [122] found that an increase in the MVD in lymph nodes correlated with the severity of the disease. In addition, HGF and its receptor c-met were overexpressed in several lymphoma cell lines [83,85]. Recent research indicated that HGF and c-met play a role in adhesion and invasion of human lymphoma cells [123,124].

Using FVIIIrAg and CD31 as endothelial markers, Schaerer et al [125] determined MVD in 18 patients with primary cutaneous B-cell lymphomas and 22 patients with cutaneous B-cell pseudolymphomas; they found that MVD was significantly different between the 2 groups. In a group of 72 B-NHL patients, Ribatti et al [126] also demonstrated that the microvessel counts in lymph nodes were higher in B-NHL than in benign lymphadenopathies, significantly higher in low-grade B-NHL compared to those in benign lymphadenopathies, and higher in intermediate-grade tumors compared to those in low-grade tumors; there was a further increase in the high-grade tumors. They also found that total numbers of both metachromatic and tryptase-reactive mast cells increased simultaneously with microvessel counts. One group reported that, besides the increased microvessel counts, there was an increase in macrophage density in B-NHL, and there is a close spatial association between microvessels and macrophages [127]. But another group found that there is no correlation between tumor MVD and response to chemotherapy in patients with diffuse large B-cell lymphomas [128]. Recently, progression of mycosis fungoides was also associated with angiogenesis [129].

4. Antiangiogenesis Therapy in the Treatment of Hematologic Malignancies

As discussed above, current data strongly suggest that the development and progression of hematologic malignancies are, at least in part, dependent on angiogenesis. In addition, a recent study demonstrated that tumor and normal endothelium are distinct at the molecular level [130]. Although they share many endothelial cell–specific markers, the endothelium derived from tumors is qualitatively different from that derived from normal tissues of the same type and is also different from primary endothelial cultures [130]. This finding may have significant implications for the development of antiangiogenic therapies.

Recently, thalidomide, an angiogenesis inhibitor, was reported to be effective in heavily treated refractory MM (Table 2) [131-151]. Thalidomide was first introduced as a sedative in West Germany in 1956 and in many other countries thereafter. By 1961, however, there were mounting reports of phocomelia and other severe congenital abnormalities associated with maternal use of thalidomide. The drug was withdrawn from the market, and its availability is highly restricted. In 1994, thalidomide was shown to be a potential antiangiogenic agent [152]. It inhibits the formation of new blood vessels from sprouts of preexisting vessels. This action may play a role in the teratogenic action of thalidomide [152].

The mechanism of thalidomide action in MM is unclear. Recent research suggested that thalidomide may or may not act directly on MM cells; its anti-MM activity may be mediated by its alteration of regulatory cytokines within the BM microenvironment, its immunomodulatory effects, or by its inhibition of angiogenesis [37,153,154]. Thalidomide has several effects on the immune system. It stimulates cytotoxic T-cell proliferation and induces the secretion of interferon γ and IL-2 by these cells [155]. It also modulates the expression of cell surface adhesion molecules [156]. Animal studies indicate that thalidomide treatment can decrease vascular density in granulation tissue [157]. A recent study demonstrated that thalidomide exerts its antiangiogenic properties via the generation of toxic hydroxyl radicals, which impair vasculogenesis and angiogenesis during embryoid-body development [158]. In addition, thalidomide inhibits microvessel formation in the rat aortic ring assay and slows human aortic endothelial cell proliferation in the presence of rat microsomes [159]. In the absence of microsomes, thalidomide has no effect on microvessel formation or cell proliferation. Nevertheless, Singhal et al [131] did not find a correlation between MVD and the response to thalidomide, suggesting that inhibition of angiogenesis may not be the major mechanism of this drug in MM. In contrast, Neben et al [132] reported that all 3 patients who had a progressive disease prior to thalidomide and achieved a partial response after 3 months of treatment showed a good reduction of blood vessel density and vascular permeability. In vitro and ex vivo studies demonstrated that thalidomide is able to induce apoptosis of myeloma cells in responding patients [160,161], whereas another investigation demonstrated that thalidomide did not induce apoptosis in myeloma cell lines or BM samples from MM patients [162]. One study demonstrated that thalidomide acts directly on MM cells, and, at least in part, inhibits IL-6 production [161].

Recently, several preliminary studies showed that thalidomide was also effective in refractory leukemia, MDS, and Philadelphia chromosome–negative myeloproliferative disorders [163-165]. However, 1 study demonstrated that thalidomide was ineffective in AML/MDS cases with poor prognoses [166]. Using inhibitors of angiogenesis can prevent acquired drug resistance. Boehm et al [167] recently reported that chronic, intermittent endostatin therapy for 3 different mouse tumors did not lead to any traces of acquired resistance. In contrast, standard chemotherapy using maximum doses of cyclophosphamide resulted in partial resistance by the third cycle of treatment and complete resistance by the fourth cycle. Recently, several agents that have been used in the treatment of leukemia were found to inhibit angiogenesis. Mantell et al [168] reported that 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] significantly inhibited VEGF-induced endothelial cell sprouting and elongation in vitro in a dose-dependent manner and also inhibited the formation of networks of elongated endothelial cells within 3-dimensional collagen gels. Roboz et al [169] reported that arsenic trioxide (As_2O_3) inhibits VEGF-induced capillary tubule formation and decreases leukemic cell VEGF production. These findings suggest that As_2O_3 may exert its antileukemic effect in part through inhibition of angiogenesis. Interestingly, at very low doses (0.1-1 pmol/L), vinblastine has striking and revers-

Table 2.
Thalidomide in the Treatment of Multiple Myeloma

Authors	Patients No.	Patients Median Age (Range), y	Dosage, g/d	Response rate, %	Reference
Singhal et al	84		0.2-0.8	32	[131]
Neben et al	11	53 (37-72)	0.4	27	[132]
Juliusson et al	19	61 (44-78)	0.2-0.8	79	[133]
Raza et al*	35	58 (37-74)	0.2-0.8	71	[134]
Mochler et al*	42		0.1-0.8	87	[135]
Durie et al	33	(36-77)	0.05-0.4	24	[136]
Rajkumar et al*	16	64 (48-85)	0.2-0.8	25	[137]
Schiller et al*	8	50 (39-70)	0.2-0.8	50	[138]
Desikan et al	180	58 (35-80)	0.2-0.8	36	[139]
Weber et al	44		0.2-0.8	25	[140]
Chen et al	8	58 (45-75)	0.2	62	[141]
Wu et al	6	62.2 (57-68)	0.2-0.8	67	[142]
Kneller et al	17		0.2-0.8	59	[143]
Palumbo et al*	77	65	0.1	76	[148]
Yakoub-Agha et al	83		0.05-0.8	66	[149]
Rajkumar et al*	42		0.2-0.8	62	[150]
Barlogie et al	169		0.2-0.8	36	[151]

*Thalidomide was used in combination with other drugs.

ible effects in vitro on certain cell functions strictly correlated with angiogenesis and in vivo on angiogenesis itself, without nonspecific cytotoxic or necrotic damage [170].

Some biological agents having the antiangiogenesis capacity were also found. Yao et al [171] demonstrated that the angiogenesis inhibitors vasostatin and IL-12 halted the growth of human Burkitt's lymphoma. In addition, Cervenak et al [172] reported that induction of human or viral IL-10 genes into Burkitt's lymphoma cells markedly reduced their ability to grow as subcutaneous tumors in SCID mice and that recombinant human IL-10 abolished and viral IL-10 reduced VEGF-165–induced neovascularization. Bertolini et al [173] reported that when endostatin was given after chemotherapy or anti-CD20 therapy, it effectively induced NHL stabilization. In animal models of high-grade NHL, they demonstrated that green tea could inhibit angiogenesis and induce endothelial and tumor cell apoptosis [174].

5. Conclusion

Recent years have seen much progress in the field of angiogenesis. There is increasing evidence indicating that angiogenesis plays an important role in the development and progression of hematologic malignancies. The successful management of patients with refractory MM with the angiogenic inhibitor thalidomide strongly supports this hypothesis. Although knowledge about the relationship between angiogenesis and hematologic malignancies is still at a very early stage, the information that has been generated thus far suggests a new way to study the pathophysiology and treatment of hematologic malignancies.

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09/22/03 12:10 INFO 5 011

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ANTI-CANCER DRUGS

Volume 13 Number 4 April 2002

MINIREVIEW PAPERS

Isothiocyanates: mechanism of cancer chemopreventive action
PJ Thornalley

Treatment of myeloma: recent developments
S Zweegman and PC Huijgens

Tumor-specific DNA in plasma of breast cancer patients
Z-M Shao and M Nguyen

PRECLINICAL REPORTS

Identification of phenyl-pyridine-2-carboxylic acid derivatives as novel cell cycle inhibitors with increased selectivity for cancer cells
SJ Berthel, IM Marks, X Yin, SG Mischke, L Orzechowski, G Pezzoni, F Sala and LT Vassilev

Synthesis and cytogenetic effects of aminoquinone derivatives with a di- and a tripeptide
Ch Pachatouridis, Z Iakovidou, E Myoglou, D Mourelatos, AA Pantazaki, VP Papageorgiou, A Kotsis and M Liakopoulou-Kyriakides

Absence of tumor growth stimulation in a panel of 16 human tumor cell lines by mistletoe extracts *in vitro*
G Maier and H-H Fiebig

CLINICAL REPORTS

Clinical pharmacology of the novel marine-derived anticancer agent Ecteinascidin 743 administered as a 1- and 3-h infusion in a phase I study
Ch van Kesteren, C Twelves, A Bowman, K Hoekman, L López-Lázaro, J Jimeno, C Guzman, RAA Mathôt, A Simpson, JB Vermorken, J Smyth, JHM Schellens, MJX Hillebrand, H Rosing and JH Beijnen

Influence of amifostine on toxicity of CHOP in elderly patients with aggressive non-Hodgkin's lymphoma—a phase II study
E Späth-Schwalbe, C Lange, I Genvresse, L Krüger, J Eucker, M Schweigert, O Sezer, V Budach and K Possinger

Capecitabine in patients with breast cancer relapsing after high-dose chemotherapy plus autologous peripheral stem cell transplantation—a phase II study
A Jakob, C Bokemeyer, S Knop, M Schupp, F Mayer and L Kanz

Dihydropyrimidine dehydrogenase-related enzymes predict efficacy and adverse reactions of UFT+cisplatin neoadjuvant chemotherapy for gastric cancer
N Takiguchi, K Koda, H Ooshima, K Oda, H Suzuki, R Ishii and M Miyazaki

Goserelin as ovarian protection in the adjuvant treatment of premenopausal breast cancer: a phase II pilot study
F Recchia, G Sica, S De Filippis, G Saggio, M Rosselli and S Rea

Docetaxel does not impair cardiac autonomic function in breast cancer patients previously treated with anthracyclines
E Ekholm, V Rantanen, K Syvänen, J Jalonen, K Antila and E Salminen

Local injection of M-CH combined with i.p. hyperthermic hypo-osmolar infusion is an effective therapy in advanced gastric cancer
Y Huang, A Hagiwara, W Wang, G Su, X Qi, D Ma and Y Fan

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Minireview paper

Treatment of myeloma: recent developments

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Melphalan was the first described treatment for patients with multiple myeloma in the 1960s and is still being used in clinical practice. However, the use of melphalan in combination with prednisone resulted in a median survival of only 2–3 years. Therefore, the dose of melphalan has been intensified since then (140–200 mg/m²). In order to diminish treatment-related morbidity and mortality due to severe myelosuppression induced by these regimens, high-dose melphalan is currently supported with autologous stem cells. Indications for high-dose therapy and the role of further intensification by performing second or allogeneic transplantations are discussed. Furthermore, new therapeutic modalities, such as inhibitors of angiogenesis, also showing direct antiproliferative, cytokine-related and immunomodulatory effects on plasma cells (thalidomide and its newer deratives), inhibitors of the transcription factor NF-κB (proteasome inhibitors) and immunotherapy are described. [© 2002 Lippincott Williams & Wilkins.]

Key words: Allogenic transplantation, melphalan, multiple myeloma.

Introduction

Multiple myeloma (MM) belongs to the plasma cell disorders, characterized by expansion of monoclonal plasma cells producing heavy and/or light chain immunoglobulins (M-protein). IgG is produced in more than 50% of patients, IgA in 30% and IgD, IgM or IgE rarely. Excretion of light chains in the urine is found in about 60% of patients. The diagnosis of MM is based on a classic triad of an increased number of plasma cells in the bone marrow (above 10%) or histologic proof of a plasmacytoma and one of the following criteria: M-protein in serum, monoclonal light chains in urine or the presence of lytic bone lesions. The clinical symptoms are caused by skeletal destruction and by bone marrow infiltration compromising normal hematopoiesis. Furthermore, a high-level of M-protein can cause hyperviscosity, renal failure and neuropathy. Suppression of production of normal immunoglobulins leads to a high incidence of infectious events.

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Epidemiology and etiology

MM accounts for 1% of all malignancies in Caucasians and 2% in US Blacks.[1] In the Netherlands there are about 2000 patients suffering from MM, of whom 50% are younger than 65 years at the moment of diagnosis.[2] The annual incidence in the Netherlands in 1995 was 4.9 in males and 3.6 in females.[3]

The most established risk factors for development of MM are acute exposure to high doses of radiation or chronic exposure to lower doses of radiation.[2] Many studies have examined the relationship between chemical exposure and MM, especially among agricultural workers. Various chemicals were found to increase the risk of developing MM 3- to 4-fold, such as consumption of dioxin-contaminated fish and proximity to dioxin-contaminated water sources.[4,5] More recently, the role of viral infections in initiating a clonal idiotypic expansion of plasma cells has been investigated. An increased risk of MM in HIV-infected patients has been described.[6] Furthermore, a role of human herpes virus-8 (HHV-8) in the onset of MM has been proposed.[7–9] However, other groups failed to detect HHV-8 sequences in bone marrow biopsies.[10,11] Recently, the presence of HHV-8 was shown to be relatively common in healthy donors, which does not support a role in MM.[12] Therefore, there is no proven link between viral infections and the development of MM.

Currently, a multi-step pathogenesis of MM is proposed. It is hypothesized that during specific DNA modification processes in B cell development Ig H

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translocations occur, resulting in new fusion genes, leading to clonal expansion of cells. Subsequently, karyotypic instability and secondary translocations occur.[13]

Prognostic factors

Prognostic factors were defined in previous trials concerning conventional treatment strategies. From recent trials investigating the role of high-dose chemotherapy with stem cell support, it was learned that the presence of previously defined risk factors was also predictive for the effect of high-dose chemotherapy. Therefore, prognostic factors, as described in the following paragraphs, should be implicated in defining the best treatment strategy for individual patients.

Staging system proposed by Salmon and Durie

Salmon and Durie proposed a staging system in 1975 based on the correlation between clinical presentation and plasma cell burden. Patients are divided in three groups according to the level of hemoglobin, serum calcium, level of M-protein and the presence of skeletal lesions (stage I, II and III). Using conventional therapy, patients with stage III disease have a median survival of 2 years, whereas patients with stage I disease frequently survive more than 5 years.[14]

Plasma Cell Labeling Index (PCLI)

In several studies the PCLI has been found to be an independent risk factor.[15–19] In this assay the number of proliferating cells is determined by the use of bromodeoxyuridine, which is only incorporated in S phase. The use of this assay is hampered by not being incorporated in daily laboratory practice.

β_2-microglobulin and albumin

Levels of β_2-microglobulin predict survival in conventionally treated as well as transplanted MM patients.[18,20,21] In conventionally treated patients, according to studies of the Southwest Oncology Group, the median overall survival (OS) times could be determined combining β_2-microglobulin levels with albumin levels (ranging from 16 months in patients with β_2-microglobulin >5.5 mg/l and albumin <30 g/l to 58 months in patients with β_2-microglobulin <2.5 mg/l and albumin >30 g/l).[22]

Similarly, in a recent performed randomized trial in which conventional chemotherapy was compared with autologous stem cell transplantation a low β_2-microglobulin level independently predicted a complete or very good partial response in both groups.[23]

Chromosomal abnormalities

Chromosomal analysis became incorporated in the diagnosis and monitoring of a variety of hemato-oncological diseases. Recently its use was introduced in patients with MM. Using conventional cytogenetic analysis, in a high proportion of patients (43%) a variety of chromosomal abnormalities, such as numerical abnormalities, deletions and translocations were observed.[24] Some of these abnormalities were found to be of prognostic significance.[25,26] Abnormalities of 11q and partial or complete deletions of chromosome 13 were associated with a worse overall as well as event-free survival. Patients with abnormalities of both chromosome 11 and 13 had an OS of only 12 months.[26] The absence of abnormalities of chromosome 11 and 13 significantly affected the duration of complete remission (CR) in patients undergoing autologous stem cell transplantations (more than 69 months in patients without chromosome 11 or 13 abnormalities versus 26 months in patients with these abnormalities), which correlated with a prolonged event-free survival and OS.[27] Analysis of their extended study of 1000 patients who underwent high-dose therapy, especially the absence of chromosome 13 abnormalities was significantly correlated with a better survival.[28] Therefore, an unfavorable karyotype is currently defined as abnormalities of chromosome 13 and/or 11.

Combination of cytogenetics with conventional risk factors

In the Total Therapy programme of the University of Arkansas, in which patients are autotransplanted twice, OS is independently correlated with the number of risk factors. Risk stratification occurred by using β_2-microglobulin levels (>2.5 versus <2.5 mg/l), chromosome abnormalities (abnormalities of chromosome 11 and 13 versus absence of these abnormalities) and duration of prior therapy (>12 versus <12 months). Event-free survival varied from median 37 (no risk factors) to 7 months (three risk factors) (Table 1).[29]

Table 1.

No. of risk factors	Median event-free survival (in months)
0	37
1	26
2	14
3	7

Treatment

Conventional induction chemotherapy

Melphalan was the first described treatment for MM in the 1960s and has remained the mainstay of therapy since then. In 1969 addition of prednisone was found to improve outcome, with a median survival of about 2–3 years.[30,31] Efforts have been made to improve outcome by using various agents and combinations for induction and maintenance therapy. On the presumption that alternating use of chemotherapeutic drugs with different mode of actions would be more effective, other alkylating agents like cyclophosphamide and carmustine, were combined with adriamycin and prednisone with or without vincristine (VCAP, VBAP and CAP). These combinations were compared with regimens in which melphalan was combined with vincristine, cyclophosphamide and prednison (VMCP).[32] Although addition of vincristine improved the response rate, recent analysis showed no differences in long-term survival. Comparison of these regimens with standard melphalan/prednisone (MP) revealed a better median survival of VMCP/VCAP and VMCP/VBAP over standard MP (36 versus 25 months); however, again there was no effect on long-term survival.[22,33,34] The advantage of newer combination therapy over MP in median survival could not be confirmed by others.[35–38] A meta-analysis on 18 published trials with 3814 patients showed that standard MP and other combination therapeutic regimens are equally effective.[39,40] Therefore, MP is still standard induction therapy.

Nevertheless, current OS rates obtained with conventional chemotherapy require new forms of therapy. In older patients improving OS will merely be dependent on improving salvage treatment and supportive care. This was recently supported by a retrospective study showing response percentages of about 50% and median remission durations of about 20 months, independent on the type of induction therapy (MP versus combination chemotherapy). OS, however, was different in the period of 1983–1986 compared to the period of 1986–1994 (33 versus 43.2 months), suggesting a role for salvage chemotherapy and supportive care.[41]

Salvage chemotherapy for relapsing MM after conventional therapy

First evidence for the efficiency of vincristine, doxorubicin and dexamethasone (VAD) as salvage therapy for patients refractory to alkylating agents came from Barlogie,[42] showing remissions in about 30% of primary resistant patients and in about 65% of relapsing patients, which was confirmed by others.[43,44] Originally, vincristine (0.4 mg/day) and doxorubicin (9 mg/m^2/day) were administered as a 4-day continuous infusion via a central venous catheter. Recently, it was found that vincristine and doxorubicin could be administered as rapid infusion, thereby bypassing the need for admission to the hospital.[45,46] In primary resistant disease similar responses were reached with solitary treatment with dexamethasone. In relapsing patients VAD was found to be somewhat more effective than dexamethasone alone.[47,48]

High-dose chemotherapy with stem cell support in patients aged less than 65 years

To improve OS in younger patients several groups explored much higher doses of melphalan with some source of stem cell support, which led to autologous stem cell transplantation becoming standard therapy in younger patients.

Autologous stem cell transplantation

Single transplants. The Royal Marsden Group was the first to explore high-dose melphalan (HDM) (140 mg/m^2) in patients with resistant disease or high-risk untreated disease. The dose of melphalan was indeed found to correlate with higher CR rates and response rates. However, the high CR rate of 32% was reached at the cost of a high treatment-related mortality rate of 16%, due to severe myelosuppression.[49] Therefore, HDM was subsequently supported with stem cells. Several groups have now treated MM patients with HDM with stem cell support after induction chemotherapy consisting mainly of VAD-like regimens. Although these phase II studies describe heterogeneous patient populations, conditioning regimens and definitions of response, progression-free survival, disease-free survival, as well as OS appear to be significantly better in

patients treated with HDM as compared with historical control groups treated with conventional chemotherapy.[50–53] Only in the study of Bladé, patients who were eligible for HDM, but did not receive it, had the same survival as compared to patients receiving high-dose chemotherapy.[54] The first prove that HDM was indeed superior as compared to conventional therapy came from the randomized study of the Intergroupe Myeloma Française.[23] CR was reached in a significantly higher percentage of patients treated with HDM compared to with conventional therapy, resulting in a better OS (for all patients: CR, 22 versus 5% and 5-year OS, 52 versus 12%; for patients aged less than 60 years, 5-year OS; 70 versus 18%).[23]

Double transplants. In view of the fact that HDM with stem cell support resulted in higher CR rates, it was hypothesized that further dose intensification would improve CR percentages even more and would be translated in better clinical outcome. In the Total Therapy program of the University of Arkansas MM patients were double transplanted using HDM (200 mg/m^2) as a first conditioning regimen and total body irradiation and cyclophosphamide as a second conditioning regimen. It was shown that CR rates increased during treatment, from 15% after induction therapy, 26% after the first to 41% after the second transplant.[27] The increase in CR percentages after the second transplant has also been shown by Harousseau,[55] Bjorkstrand,[56] Weaver[57] and ourselves.[53] In view of the hypothesis that achievement of a CR results in a longer event-free survival and better OS, double-transplant programmes might be shown to be more effective than single transplants in MM patients. Again from the Intergroupe Myeloma Française there came indications that higher CR rates obtained by double transplantations are indeed translated into a better OS. Although an earlier performed analysis of their randomized study comparing single transplantation (HDM 140/TBI) with double transplantation (HDM 140 followed by HDM140/TBI), only showed an advantage in OS in low risk patients with a β_2-microglobulin of below 3 mg/l,[58] a recent interim analysis showed that the significantly higher percentage of CRs and very good partial remissions in double-transplanted patients as compared to single-transplanted patients was not limited to low risk patients (61 versus 50%). After the first randomization (single versus double transplantation) a second randomization occurred (bone marrow versus peripheral stem cells). The 5-year post-second randomization survival was significantly higher in patients transplanted twice with the use of peripheral stem cells compared to patients transplanted once or transplanted with bone marrow (60 versus 40% for patients transplanted with peripheral stem cells, 43 versus 35% for patients transplanted with bone marrow). A second transplant could be performed in 75% of patients with a first transplant.[59] However, to determine the role of double transplantation, the final results of this and other randomized studies have to be awaited.

Allogeneic stem cell transplantation

Although CR rates and OS increased after the introduction of autologous transplantation protocols compared with conventional chemotherapy, all patients eventually relapse and molecular remissions are rare. Considerably higher percentages of molecular remissions have been described after allogeneic transplantations. Using clonal markers based on rearrangement immunoglobulin heavy chain genes, in allogeneic transplanted MM patients in complete clinical remission, molecular remission rates of 27–75% have been reported.[60–62] These percentages were higher as compared to autologous transplanted MM patients (up to 16%).[60,61] Moreover, patients who achieved a molecular remission had significantly lower relapse rates. Therefore, allogeneic transplantation might potentially cure patients.

However, in clinical practice allogeneic transplantation procedures were found to be complicated by high transplantation-related mortality rates up to 50%.[62–67] This is probably accounted for by inclusion of heavily pretreated MM patients and patients with refractory disease. The analysis performed by the European Group for Blood and Marrow Transplantation centers supports this hypothesis, as a significant reduction in transplantation-related mortality was found comparing results obtained during the period of 1983–1993 with 1994–1998 (38 versus 21%). Reasons for this decline were better results of transplantation earlier in the disease and better supportive care. Reduction in transplantation-related mortality was translated in a higher median OS; 10 months for patients transplanted in the earlier time period versus 50 months during the later period.[68]

Another strategy to diminish transplantation-related mortality may be performing allogeneic transplantation following treatment with non-myeloablative regimens instead of myeloablative regimens. The rationale is that the advantages of allogeneic transplatations are thought to be the

result of a graft versus myeloma (GVM) effect. Proof for that came from studies in which infusion of donor lymphocytes (DLI) resulted in partial and even complete clinical responses.[69,70] Therefore, in order to diminish direct toxicity of the myeloablative regimen, non-myeloablative regimens consisting of melphalan alone, total body irradiation alone, combinations of fludarabin, anti-thymocyte globulin and busulfan were explored.[71–73] In the studies of Badros *et al.*[71] and Garban *et al.*[73] patients were previously autotransplanted. Response rates of 75% were observed, of which 30% were CR. Even in patients with a refractory relapse remissions were observed. Transplantation-related mortality was found to be minimal.

However, it is currently unknown which patients will benefit from up-front allogeneic transplants, because of the lack of randomized protocols comparing autologous versus allogeneic transplantations. Only one retrospective case-matched study, describing patients treated during 1983–1994, compared the outcome of allogeneic transplants versus autologous transplants. It was found that OS was significantly better in autologous transplanted patients. This was due to the already mentioned high transplantation-related mortality of 41% in allogeneic transplanted patients,[64] which is expected to decline when transplanting patients up-front or using non-myeloablative regimens. This is not supported, however, by preliminary results obtained from the Dutch HOVON 24 study in which MM patients were transplanted allogeneic up-front. Median OS appeared to be worse in patients transplanted allogeneic as compared to patients transplanted autologous (21 versus 45 months).[74]

Maintenance therapy with interferon (IFN) after conventional therapy

Most studies in which the efficiency of treatment with IFN-α was investigated showed a improvement of remission durations of 6–9 months. However, in most studies this was not translated into an improvement of OS.[75–79] A recent meta-analysis concluded: 'the survival benefit, if any, is small and needs balancing against cost and toxicity'.[80]

Maintenance therapy with IFN after high-dose chemotherapy

In transplanted patients the role of IFN as maintenance therapy has been less extensively studied. Cunningham reported a significantly better OS at 4.5 years, which, however, ceased to exist at 7.5 years.[81] In a retrospective analysis of the EBMT registry, OS was better in patients treated with IFN. This analysis, however, was hampered by differences in prognostic factors between treated and untreated patients, and by the fact that only patients in CR or partial remission at 6 months after transplantation were analyzed.[82] Therefore, the value of interferon as maintenance therapy after high-dose chemotherapeutic regimens has yet to be determined.

New therapeutic modalities

Thalidomide

Several studies have shown an increased microvessel density in the bone marrow of MM patients, suggesting a role for angiogenesis in MM.[83–85] Thalidomide, originally a sedative which had become obsolete because of teratogenicity, was found to inhibit angiogenesis.[86] The first evidence for clinical efficacy of thalidomide in patients suffering from MM came from a study by the group of Barlogie, in which 89 refractory or relapsed MM patients showed a response rate of 32%.[84] Since then several studies have shown effectiveness of thalidomide in relapsed and refractory patients, with response rates varying up to 64%.[87,88]

The efficacy of thalidomide appeared not to be solely the result of inhibiting angiogenesis, as no correlation was found between microvessel density and response to therapy.[84] Therefore, research was focused on other mechanisms whereby thalidomide could affect the growth of plasma cells. First, thalidomide was found to diminish the expression of the adhesion molecule ICAM-1. As a consequence, less plasma cells adhered to stromal cells, resulting in inhibition of growth and survival.[89] Second, thalidomide was found to block the effect of cytokines important to growth of plasma cells [interleukin (IL)-6, tumor necrosis factor-α and IL-1].[90] Third, production of IFN-γ and IL-2 by cytotoxic T cells and natural killer cell number and function was enhanced by thalidomide.[91,92] Furthermore, research on the cause of teratogenicity of thalidomide showed free radical-mediated oxidative DNA damage.[85] In summary, thalidomide has proven to be effective in relapsed and refractory MM patients, although the mechanism is still unclear.

Future role of thalidomide

The value of combining thalidomide with other established treatment modalities for MM is currently

under investigation. There are already indications that the combination of thalidomide and dexamethasone is more effective than dexamethasone alone in patients relapsing after autologous stem cell transplantations (57 versus 27% response rate, respectively).[93] Also, in patients with a lack of continuing response to thalidomide alone or patients with a rash on thalidomide, addition of low-dose dexamethasone (4 mg) further reduced M-protein levels and was associated with fewer side effects.[94] The role of thalidomide in newly diagnosed patients, undergoing high-dose chemotherapy, in induction as well as maintenance therapy will be investigated in several protocols. For example, in the Netherlands the HOVON 50 protocol has started—a randomized study in which the value of thalidomide in the induction phase and in maintenance phase will be investigated. All patients will receive one or two courses of high-dose chemotherapy with stem cell support. Since a tendency to venous thrombosis has been reported, patients in the thalidomide arm will receive prophylactic low-molecular-weight heparin (LMWH).[93,95]

New thalidomide derivatives

New analogs [3-amino-phthalimido-glutaramide (S-3APG)] and derivatives (CC5013) of thalidomide are currently being investigated in *in vitro* (S-3APG) or *in vivo* (CC5013) phase I studies. *In vitro* and animal studies show better direct antiproliferative, cytokine-related and immunomodulatory effects against MM cells than thalidomide. Preliminary studies using CC5013 in MM patients show anti-tumor activity (greater than 25% response in 15 of 24 patients) with acceptable toxicity, mainly myelosuppression, but no somnolence and neuropathy as described in patients using thalidomide.[96,97]

Immunotherapy

Although immunotherapy has been reported in several meetings, abstracts and some reports, its usage at a large scale will probably not take place for several years.

Passive immunotherapy using monoclonal antibodies (mAb)

Plasma cells are known to express several antigens. Until now, however, no one antigen has been shown to be ultimately specific. CD20 is expressed on B cells from pre-B cells to mature B lymphocytes. On plasma cells, weak expression of CD20 was found to be present in 0–20%.[98–100] There might be a role for anti-CD20 mAb in selected patients with CD20-expressing plasma cells. At the moment the only preliminary results of clinical trials using a mAb against CD20 (rituximab) were described by Treon *et al.*[101] Among 18 treated patients, one experienced a partial response and five were reported to have stable disease. A patient with light chain disease was previously reported by the same authors, showing a partial response after one course of rituximab.[102]

Another candidate for mAb directed therapy might be CD138 (syndecan-1), which is a heparan sulfate proteoglycan, expressed on plasma cells. CD138 is also expressed on normal plasma cells, epithelial and endothelial cells, leading to cross reactivity. mAbs may be raised against specific regions within syndecan-1. As compared to syndecan-1 expression on normal cells, syndecan-1 on malignant plasma cells is underglycosylated, thereby uncovering epitopes which are normally not reachable for mAb, creating a possibility for therapeutic intervention.[103–105]

Vaccination therapy

Each malignant plasma cell clone has its own tumor-specific antigen, as each clone produces a specific monoclonal immunoglobulin (idiotype). Therefore, MM is an ideal candidate for anti-idiotype vaccination in order to generate a specific cytotoxic T cell response. Tumor-specific immune responses have been described in clinical practice, using vaccination with conjugated idiotypes or idiotype-primed dendritic cells obtained from leukapheresis material of patients.[106–111] Even shortly (2–4 months) after high-dose chemotherapy and peripheral stem cell transplantation the immunocompetence of patients was good enough to generate an immune response. Moreover, in some of these patients an idiotype-specific T cell proliferative response was observed.[108–110,112] It has been suggested that the ability to generate an anti-idiotype response is related to the extent of remission. The absence of a circulating M-protein might favor immune responses, as circulating immunoglobulins can induce anergy or deletion of idiotype specific T cells.[110,113] Specific cytotoxic T lymphocytes can also be generated *ex vivo* either by activated autologous plasma cells or by autologous dendritic cells and subsequently reinfused into the patients.[114]

Clinical use of vaccination therapy will be hampered by the fact that plasma cells do excrete immunoglobulins, thereby losing expression and furthermore by high levels of circulating immunoglobulins. It is therefore thought that the use of vaccination therapy will be especially effective in the setting of minimal residual disease.

NF-κB as a therapeutic target for MM

NF-κB is a transcription factor which upregulates the expression of IL-6, vascular endothelial growth factor, cell adhesion molecules and anti-apoptotic factors. Thereby activation of NF-κB confers survival potential for MM cells. It was already known that β_1-integrin-mediated adhesion of MM cells to fibronectin in the bone marrow environment resulted in drug resistance. As NF-κB activity was found to be dramatically increased in MM cells adhered to fibronectin, inhibition of signal transduction pathways initiated by cell adhesion may provide strategies to overcome drug resistance to chemotherapy. Irrespective the presence of drug resistance, inhibition of NF-κB might be a useful therapy in MM patients, as constitutive expression of NF-κB activity is present in MM cells. First indications that such intervention may be successful were presented at the American Society of Hematology meeting in December 2001. PS-341, a selective inhibitor of the proteasome, having numerous effects on regulatory proteins, including the blockade of NF-κB activation, was administered i.v. to heavily pretreated patients (including high-dose therapy), refractory to their most recent therapy. Preliminary evidence of antitumor activity was reported (52% of patients showed a response, 33% of patients had stable disease, after four cycles of PS-341).[115] Specific blockade of NF-κB activation by PS-1145 (a specific IκBα kinase inhibitor) or SN50 (a cell-permeable specific inhibitor of NF-κB nuclear translocation and activity) also exerted anti myeloma effects in *in vitro* studies. Dexamethasone, upregulating IκBá protein, was found to enhance blockade of NF-κB activation by PS-1145.[116,117]

Supportive care

Bone disease: treatment with bisphosphonates and osteoprotegerin

Almost all patients with MM will encounter skeletal events in the course of their disease. At diagnosis more than 50% of patients present with vertebral fractures and up to 30% with non-vertebral fractures due to the presence of osteolytic bone disease.[118] Furthermore, about 60% of patients have osteoporosis and 20–30% develop hypercalciema as a result of osteoclast-mediated bone resorption.[119] Osteoclast activation is thought to be the result of the production of osteoclast activating factors by plasma cells and bone marrow stromal cells. The precise nature of these factors (IL-1β, TNF-α, lymphotoxin) as well as the site of production (plasma cells or stromal cells) is still debated.[120] Recently, it was found that myeloma cells express RANKL (the ligand for receptor activator of NF-κB, also named osteoprotegerin ligand) or upregulated RANKL expression in preosteoblastic or stromal cells.[120,121] RANKL stimulates osteoclast activity and differentiation, and therefore it may be involved in the pathogenesis of MM-induced bone disease. Irrespectively of the underlying mechanisms, plasma cells are known to activate osteoclasts to produce IL-6.[120] Furthermore, plasma cell adhesion to bone marrow stromal cells initiate stromal IL-6 production.[122,123] Thereby the bone marrow environment and especially the environment created by resorbing bone is supportive for further growth of plasma cells. Therefore, it can be postulated that inhibiting osteoclasts by biphosphonates, not only leads to diminished bone resorption but, moreover, diminish proliferation of plasma cells. The effectiveness of clodronate and pamidronate in bone disease of MM patients has been observed by several, but not all, investigators. Clodronate, given orally, was found to decrease fractures in most studies.[124,125] In one study, no influence was observed on the incidence of fractures or pain, although less progression in lytic lesions was observed in the treatment arm in this study.[118] Pamidronate, given i.v., was also found to decrease the incidence of skeletal events, as well as increasing the quality of life.[126] In order to circumvent the inconvenience of i.v. administration of pamidronate during several hours, on the one hand, and the problem of poor resorption of oral biphosphonates, on the other hand, the third generation biphosphonates (zoledronate and ibandronate) which can be administered i.v. in a few minutes will probably be the best option. However, the effectiveness on clinical outcome has to be compared with conventional biphosphonates first.

Whether treatment with bisphosphonates will also affect disease progression or survival is not completely elucidated yet. In the extended above mentioned study of Berenson *et al.* a survival benefit was observed in these patients who received at least

two courses of pamidronate.[127] An anti-myeloma effect has been describes by others, *in vitro* as well as *in vivo*.[128,129]

In the future the role of osteoprotegerin, which inhibits osteoprotegerin ligand, and therefore inhibits osteoclast differentiation and activation, will probably be investigated as a new treatment option for bone disease in MM patients.[120,121]

Erythropoietin

The pathogenesis of anemia in patients suffering from MM is diverse. Inadequate production of erythropoietin led to investigating the role of epoetin α in the treatment of anemia in patients with multiple myeloma.[130] Since then several studies has been reported, showing effect in untreated patients with smoldering MM without symptoms, except for anemia,[131] in patients on chemotherapeutic treatment[132] and in patients with progressive myeloma resistant or refractory to chemotherapy.[133–135] Increases of 2 g/dl or more in Hb level (responders) were noted in about 70% of patients. In 30–50% of patients transfusions were no longer required. A recent large randomized study, describing 145 patients, confirmed earlier results: 57.6% of epoetin treated patients responded versus 9.1% in untreated patients. From epoetin-treated patients, 45.5% achieved Hb levels of more than 12 g/dl, versus 3.0% of untreated patients.[136] Furthermore, the quality of life increased during erythropoieitin treatment.[130,136]

It is not known whom to treat and how long to continue before deciding that treatment is not effective. Concerning whom to treat, patients with low endogenous erythropoietin levels were found to respond to treatment with erythropoietin. By relating erythropoietin levels to the predicted serum erythropoetin level for the degree of anemia, it could be determined that approximately 75% of patients with low levels for the degree of anemia responded to treatment with erythropoietin, whereas in patients with adequate levels only about 25% responded.[137] In the light of risks of blood transfusions and the necessity of hospital visits, it can be advocated to perform a trial in all MM patients with anemia with erythropoietin, irrespective the level of endogenous erythropoietin. Based on the maximum times to respond to treatment of about 3–4 months in most studies, such a trial of erythropoietin treatment should take about 4 months, before deciding erythropoietin treatment being not effective.[130,136,138]

Conclusi ns and futur dir ctions

Patients aged 65 years or younger

The introduction of high-dose chemotherapy with autologous stem cell support has significantly improved the outcome of patients with MM, compared to conventional chemotherapy. Therefore, upfront treatment with high-dose chemotherapy is the standard of care now. From ongoing randomized trials, it might be expected that the higher complete clinical and molecular remission rates reached with double transplant procedures will be translated in better OS. However, before becoming standard therapy the final results have to be awaited. In patients under 55 years, with high-risk multiple myeloma (i.e. with high β_2-microglobulin levels and abnormalities of chromosome 13), the role of allogeneic transplantations (after myeloablative pretransplant regimens as well as non-myeloablative pre-transplant regimens followed by donor lymphocyte infusions) has to be determined. Future trials will be directed to the role of concomitant treatment with thalidomide or its derivatives in induction and maintenance therapy, and on the effect of immunotherapy in a situation of minimal residual disease after high-dose chemotherapy.

Patients aged over 65 years

Although high-dose chemotherapy has been performed in patients older than 65 year,[139] in most centers it is current strategy to give conventional chemotherapy in older patients. Although various agents in several combinations have been investigated in the past, melphalan/prednisone has remained standard therapy. However, an OS rate of about 2–3 years necessitates exploring new forms of therapy. Thalidomide or its derivatives will probably be introduced earlier in treatment strategies, combining it with conventional chemotherapy.

New targets for therapy

Activation of NF-κB, a transcription factor, confers a survival advantage to tumor cells, including MM cells. Proteasome inhibitors, such as PS-341, block NF-κB activation and were indeed found to possess anti-myeloma effects in ongoing phase II trials. New drugs inhibiting signal transduction pathways will be explored.

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